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ABSTRACT

The methods given for collecting, preserving, and processing mosquitoes and other archropods for isolation of arboviruses are those used by the National Communicable Disease Center. Techniques of collecting mosquitoes as they bite, using light or bait traps, and from their daytime resting sites are described and illustrated. Details of subsequent storage and processing for virus extraction and identification are included. A short section describes differences in procedure when ticks or mites are collected. (AL)

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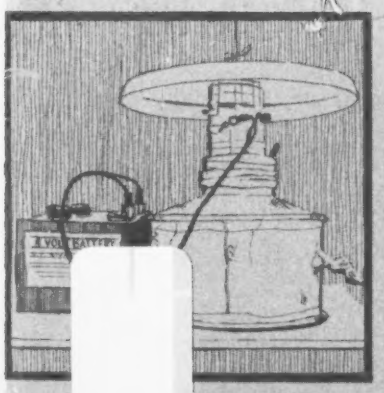

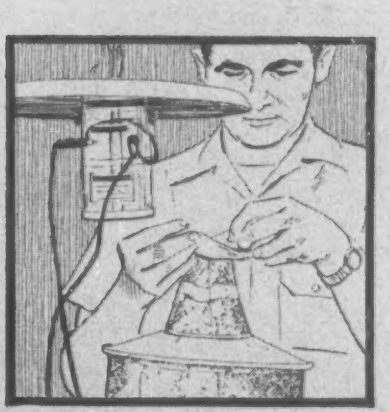

COLLECTION & PROCESSING OF MEDICALLY IMPORTANT ARTHROPODS FOR ARBOVIRUS ISOLATION

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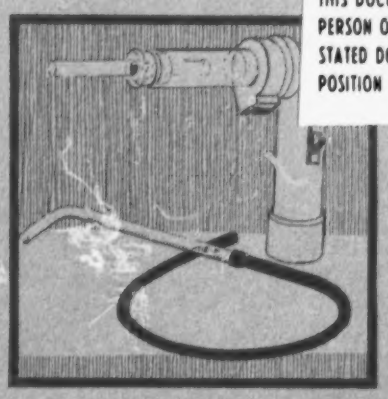

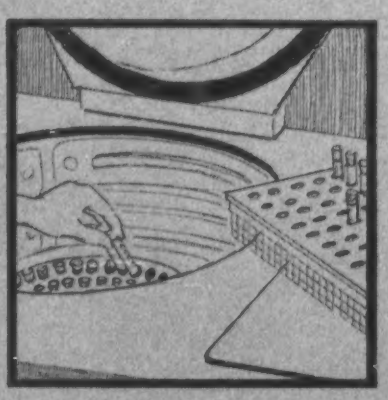

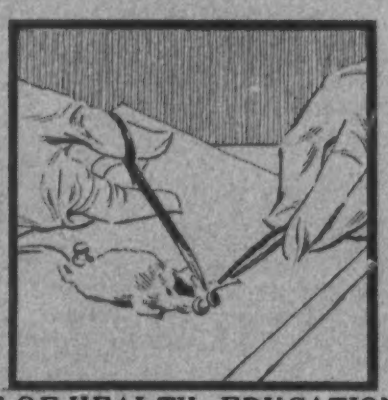
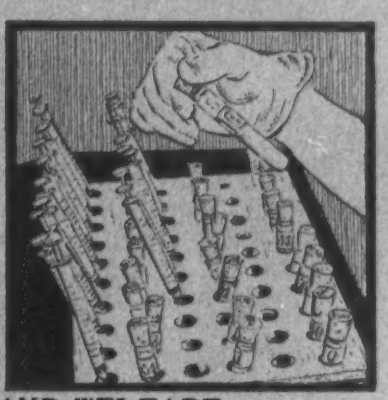

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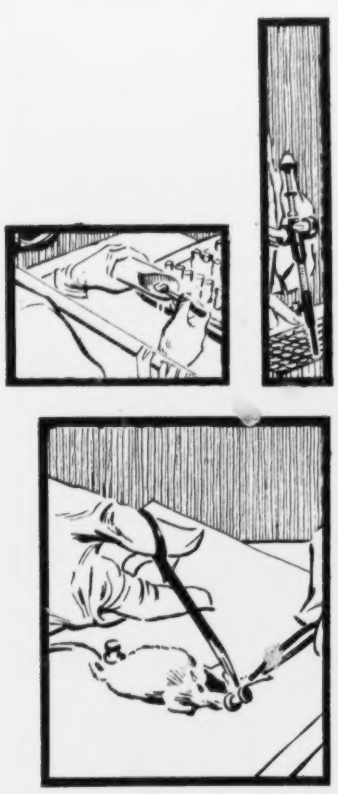

COLLECTION & PROCESSING OF MEDICALLY IMPORTANT ARTHROPODS FOR ARBOVIRUS ISOLATION

By W. DANIEL SUDIA and ROY W. CHAMBERLAIN

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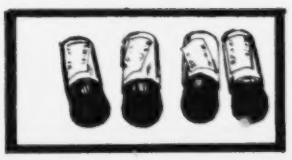
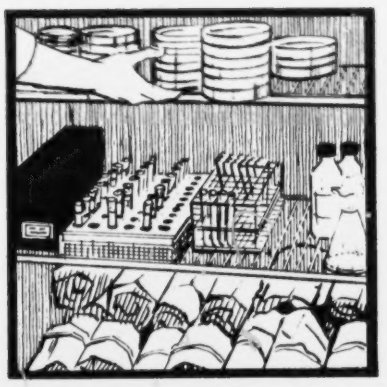
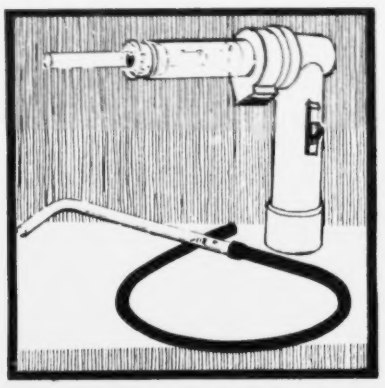
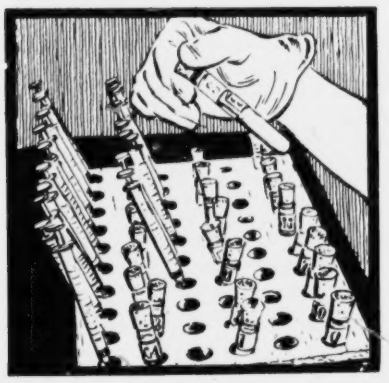
January 1967









PREFACE

This bulletin was prepared in response to numerous requests for detailed information on methods for catching, preserving and processing mosquitoes and other arthropods for isolation of arboviruses. The methods given have evolved and been tested over the past few years in the Arbovirus Vector Laboratory, Arbovirus Unit, CDC. Failure to consider special methods used by many eminent arbovirus workers from other areas does not reflect discredit upon them but rather points out the limitations of the present work.

Grateful acknowledgement is made to the many resourceful people, within and without CDC, who have directly or indirectly contributed to the development and testing of the methods herein described.

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MOSQUITOES

COLLECTION AND PRESERVATION

For arbovirus survey purposes, a great many species of mosquitoes must be taken alive in large enough numbers for significant virus tests. A variety of collecting methods may be required to produce adequate samples, since no one method will yield all species. Once collected, the mosquitoes must be handled and processed carefully to prevent the loss of the virus they may contain.

Collection with CDC light traps

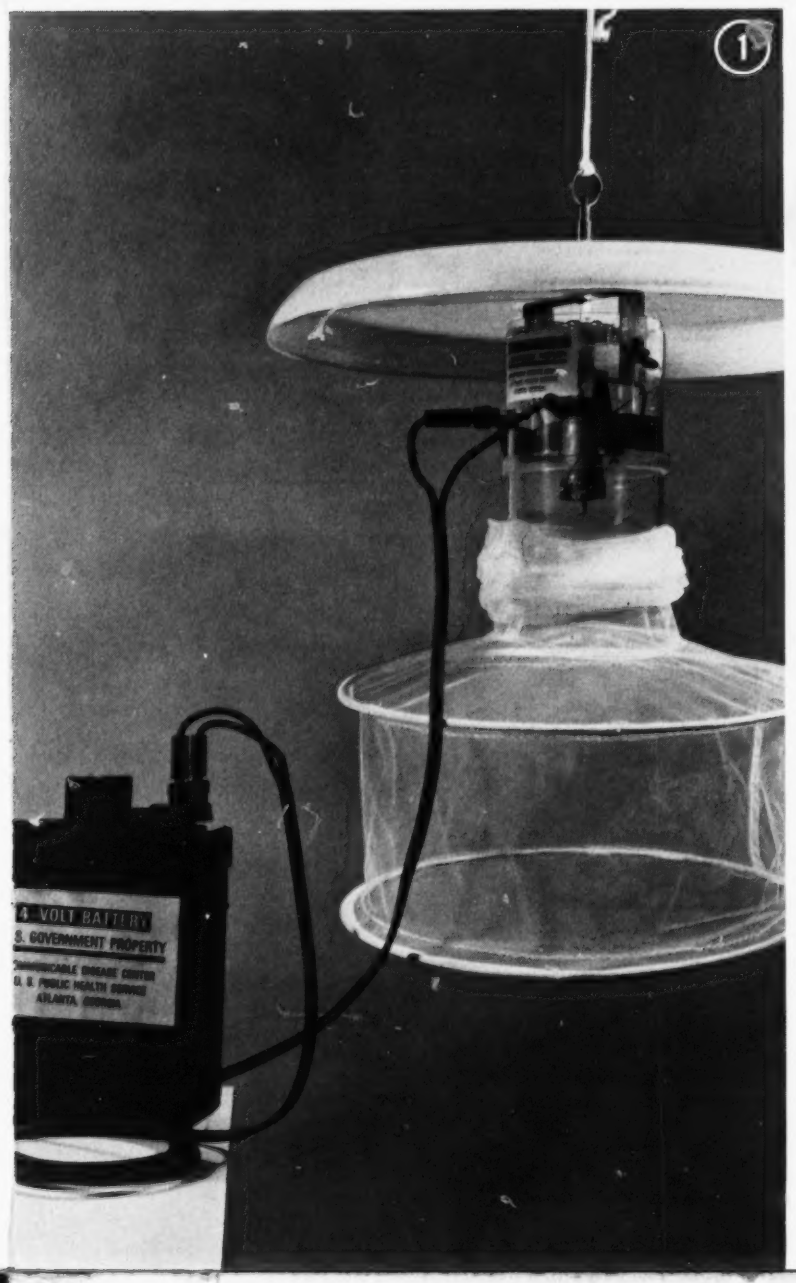
Experience has shown light traps to be a productive means of collecting mosquitoes, both in consideration of numbers of individuals captured and diversity of species represented. A miniature battery-operated light trap has been developed at CDC[1/] (Figure 1), adaptable for either a 4 or 6 volt battery. Because of its light weight and small electrical current demand, it is highly portable and can be used advantageously in even the most primitive areas. It is used extensively in many CDC field programs, supplemented when necessary by other collecting methods.

Depending upon the size and ecological diversity of the study area, and the number of mosquitoes desired from a particular site as a sample for virus testing, the traps may be clustered only 50 to 100 feet apart (Figure 2) or may be widely separated over many miles. A total of 20 to 30 traps are operated effectively by a two-man crew if placement is in reasonably accessible locations. All equipment needed may be transported in a standard station wagon (Figure 3). A man can easily carry 6 small motorcycle batteries or 12 dismantled traps at a time into the field in lightweight cases supplied with shoulder straps (Figure 4).

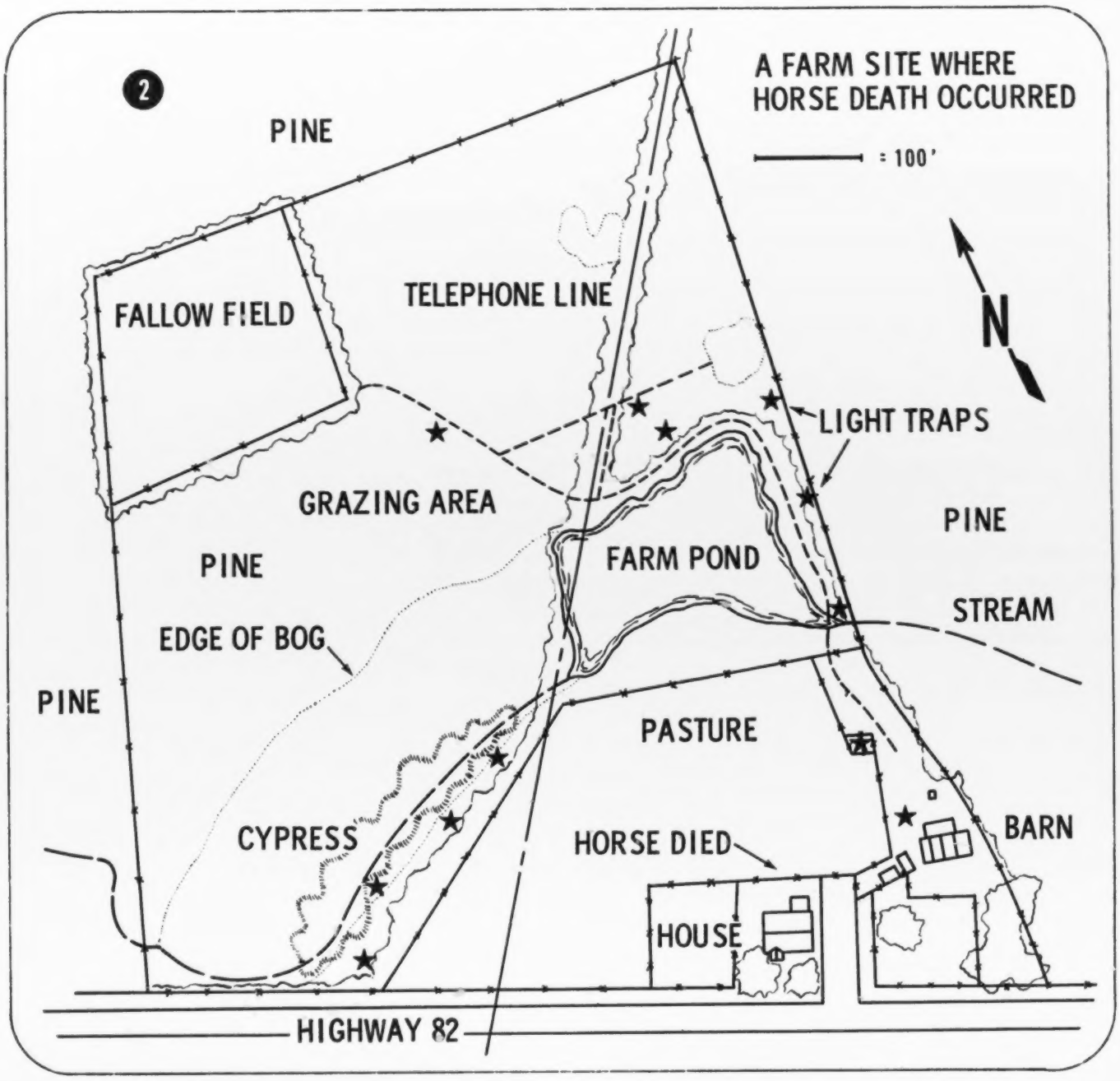
Site selection for light traps is important and improves with the experience of the collector. In general, the best catches are made where the cover is good and the humidity relatively high. Locations a short distance into the margins of woods or swamps are particularly favorable; traps hung over open water or in open pastures are, on the other hand, usually less productive. If a site fails to produce the expected number of mosquitoes, judging from collections in other traps in the area, the trap is relocated. Sometimes a shift of only a few yards makes a considerable difference in the number of mosquitoes attracted.

Care is taken to place the traps where they will be shaded from the morning sun, to increase the chance of mosquito survival.

The best light trap catches are made during the dark of the moon or on overcast nights; the weak light of the CDC trap competes poorly with bright moonlight. For this reason light-trapping is usually restricted to



1/ Sudia, W. D. and Chamberlain, R. W., 1962. Battery-operated light trap, an improved model. Mosq. News, 22:126-129.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A FARM SITE WHERE
HORSE DEATH OCCURRED
= 100'
N
PINE
FALLOW FIELD
TELEPHONE LINE
LIGHT TRAPS
GRAZING AREA
PINE
FARM POND
PINE
STREAM
EDGE OF BOG
PINE
PASTURE
CYPRESS
HORSE DIED
BARN
HOUSE
HIGHWAY 82

3

4

the darkest half of the month unless supplemented by other means of mosquito collection.

Rainfall during the night generally does not reduce the light trap catch; to the contrary, intermittent showers appear to enhance the catch. Recent studies have shown that a carbon dioxide supplement to the CDC light trap greatly increases the trap's efficiency.[2/] A 1 or 2 pound piece of dry ice is wrapped in newspaper or aluminum foil (Figure 5) and suspended immediately above or beside the trap. Greater numbers of mosquitoes are caught, the species spectrum increased, and the use of the trap less restricted as to placement and moonlight conditions. Also, if the trap is set in late afternoon, diurnal species can be captured.

The traps are visited as early as practicable the next morning. The mosquito collecting bag containing the captured mosquitoes is removed from the trap and the sleeve loosely knotted (Figure 6). The bag is then gently collapsed, labeled as to site and date (Figure 7), and placed on edge in a styrofoam picnic chill chest containing two frozen quart-sized refreezant cans (Figure 8). Mosquitoes cooled in this manner remain inactive and alive for several hours, even in summer heat, permitting the pick-up of bags at widely scattered points prior to killing and freezing the mosquitoes for storage. It is important not to crowd the collecting bags by placing too many in the chest, as the mosquitoes may be killed

2/ Newhouse, V. F., Chamberlain, R. W., Johnston, J. G., Jr., and Sudia, W. D., 1966. Use of dry ice to increase mosquito catches of the CDC miniature light trap. Mosq. News, 26 (1): 30-35.

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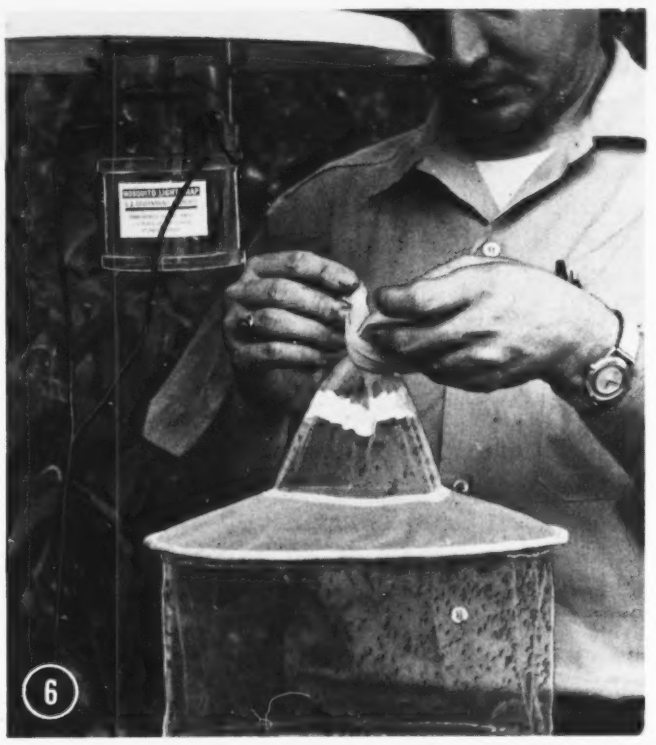
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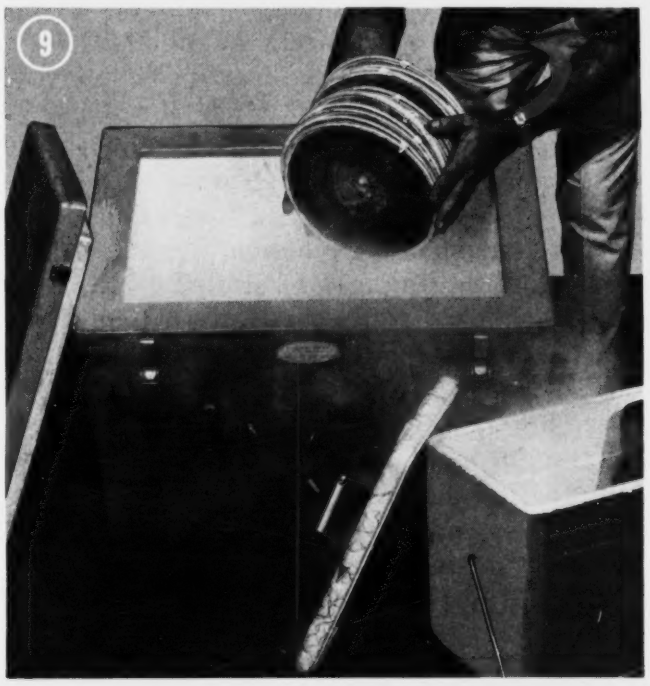
by crushing and the inside bags are not sufficiently cooled. Fifteen bags is a maximum number to be cooled by the two refreezant cans in a 1½ cubic foot chest.

The bags of mosquitoes are taken to a convenient work site (field lab, tourist court, or merely a shady place and station wagon tailgate) for rough sorting and storage. The bags are transferred to a portable (5 cubic foot) styrofoam dry ice chest and allowed to remain there about 15 minutes to kill the mosquitoes by freezing (Figure **9**). The bags are then carefully removed two or three at a time and allowed to warm up a few minutes to thaw the mosquitoes. When a bag is warmed sufficiently to open it without mosquito breakage, a gentle swinging in the air will hasten thawing of the mosquitoes (Figure **10**). If they are handled while frozen, the mosquitoes will shatter. The sleeve of the bag is untied and the thawed mosquitoes gently shaken out onto a white light-trap lid for sorting (Figures **11**, **12**). They are quickly separated (within 10-15 minutes, maximum) from the "trash" insects by aspirator and forceps, placed in flat-bottomed shell vials (17 by 55 mm) and tightly plugged with rubber stoppers (Figure **13**). The tubes receive adhesive tape labels giving the date and location, and are further sealed by wrapping the juncture of glass lip and stopper with several rounds of ½-inch waterproof adhesive tape (Figure **14**). If more than one tube is required for a single trap collection, each is numbered (e.g., 1 of 3, 2 of 3, etc.) to simplify associating them later when being processed in the laboratory. A field work-sheet is made up at the time of mosquito tubing, giving trap numbers, dates, and approximate number of mosquitoes in each catch. These data are helpful later in making decisions as to pooling and identification schedules.

The mosquitoes may be anesthetized or killed by chloroform instead of freezing, provided the chloroform is used conservatively and the mosquitoes handled as quickly as with the other method. After tubing, the mosquitoes are immediately stored on dry ice in labeled cylindrical ice-cream cartons (Figure **15**) and are later transported to the main laboratory. There they are stored in a mechanical freezer at −60° C until they are identified, suitably pooled, and tested for virus. No evidence of virus loss has been noted in mosquito specimens stored at this temperature for a year in the field tubes.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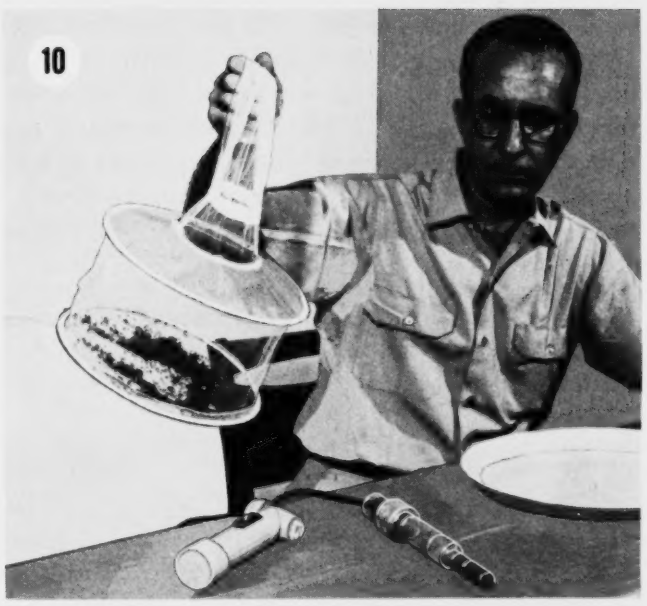
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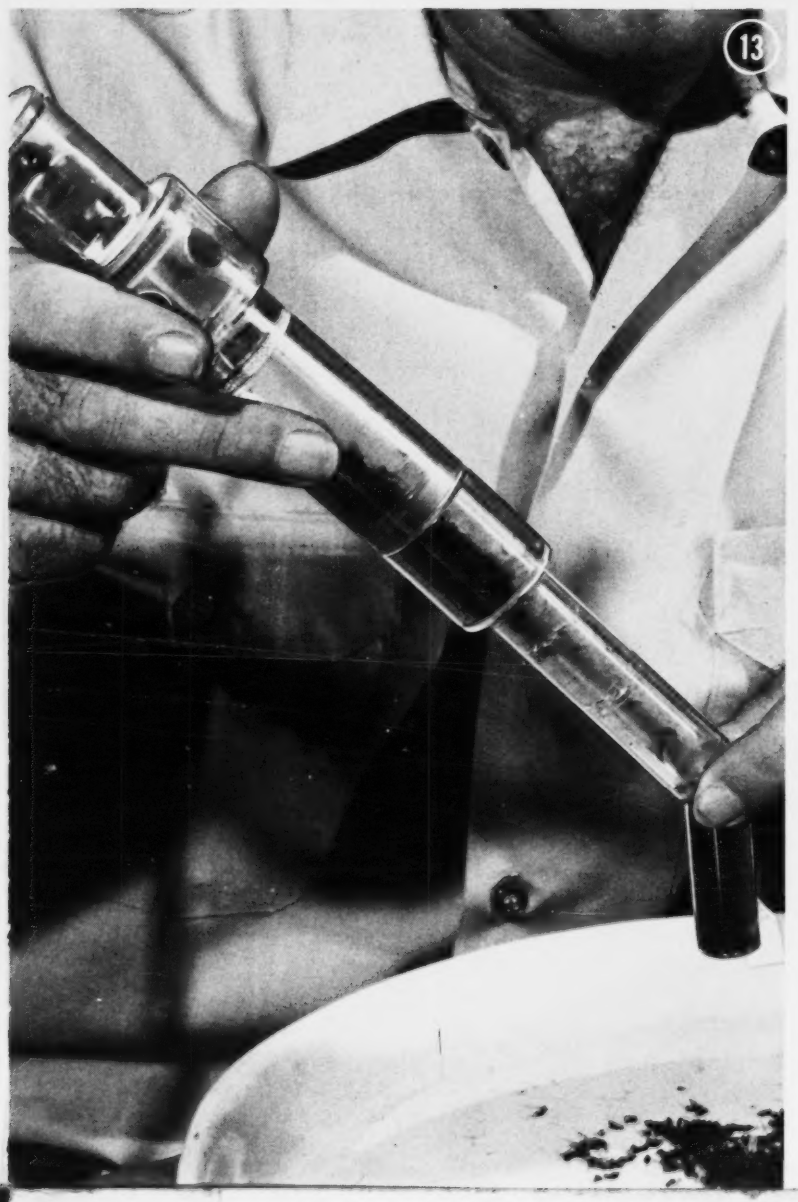


Collection from daytime resting sites

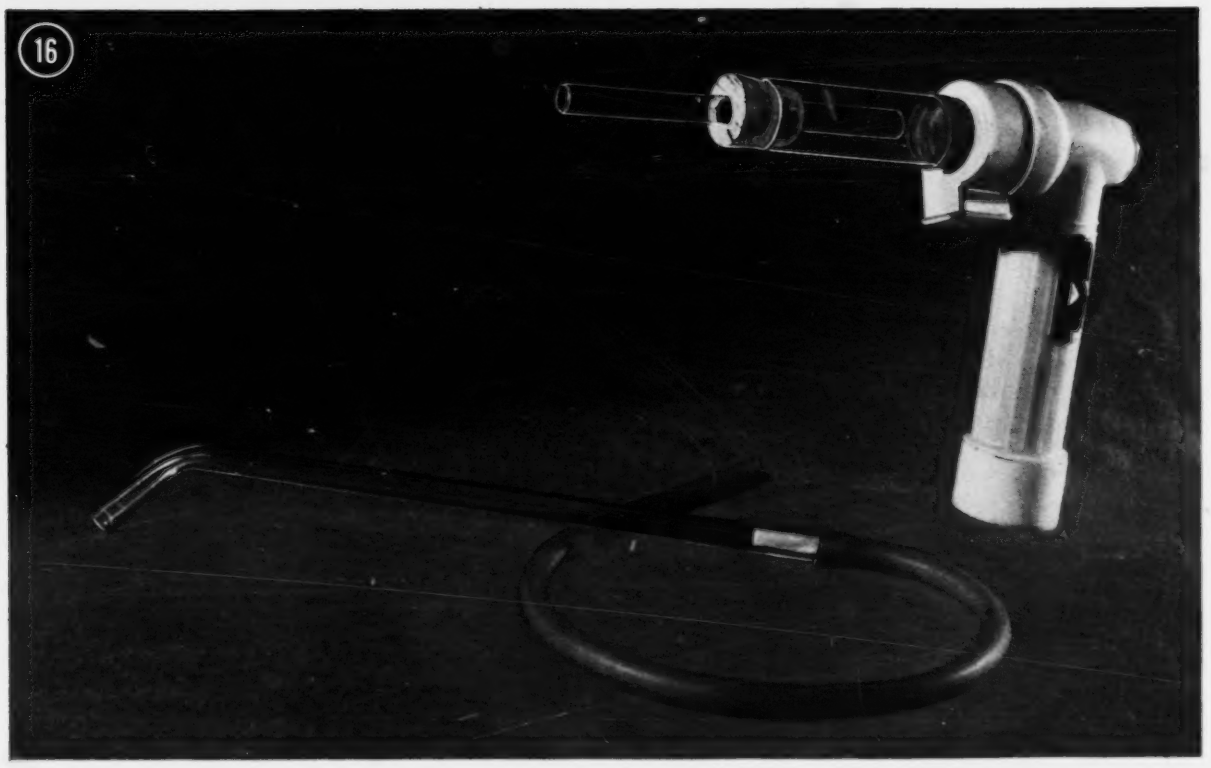
Many species of mosquitoes can be collected from various daytime resting sites, including back-yard chicken houses, outbuildings, garages, crawl spaces beneath porches, in culverts, beneath bridges, and in hollow trees and stumpholes. Under some circumstances, the inside of houses may be inspected. Specially constructed structures, such as privy-like sheds or 1-cubic-foot black or dark-red boxes, may be provided in planned studies. The resting collections are particularly useful for obtaining species which are only weakly attracted to light traps, such as *Culex pipiens Linn.* and *C. quinquefasciatus Say*, both of which are important urban vectors of St. Louis encephalitis.

In making the collections, the inside walls of chicken houses, garages or other outbuildings, close to the ceiling as well as near the floor, should be examined for resting mosquitoes since varying temperature, humidity and light conditions influence the resting position of the mosquitoes. Old spider webs are usually favorite sites. A flashlight is needed for examining the darker portion of the site, and collections are most easily made using a mouth or battery-powered aspirator (Figure 16).

A battery-powered aspirator can be adapted from a vacuum flashlight (vacuum lint brush) by removing the brush from the port and attaching a mosquito-collecting unit.[3/] Such an aspirator greatly accelerates the collection of mosquitoes without the danger and discomfort of inhaling the contaminated dust usually encountered.

If the mosquito conditions in the area under study are not already known to the collectors, a general survey is first made to locate productive collecting sites. Using a local map, the area is divided into sections for systematic inspection and collection from favorable resting places (Figure 17). In an urban epidemic area, two men are assigned to a section as a team, one to serve as driver, the other as map reader and guide. If the city is large, several teams may be required for coverage of all sections within a reasonable time. Some portions of an average city, such as well-kept residential blocks, can usually be dismissed after cursory inspection as being unfavorable for mosquito production and harborage; the older sections and outskirts, on the other hand, are generally more favorable for mosquitoes and require more time.

3/ Husbands, R. C., 1958. An improved mechanical aspirator. Calif. Vector Views, 5: 72-73.



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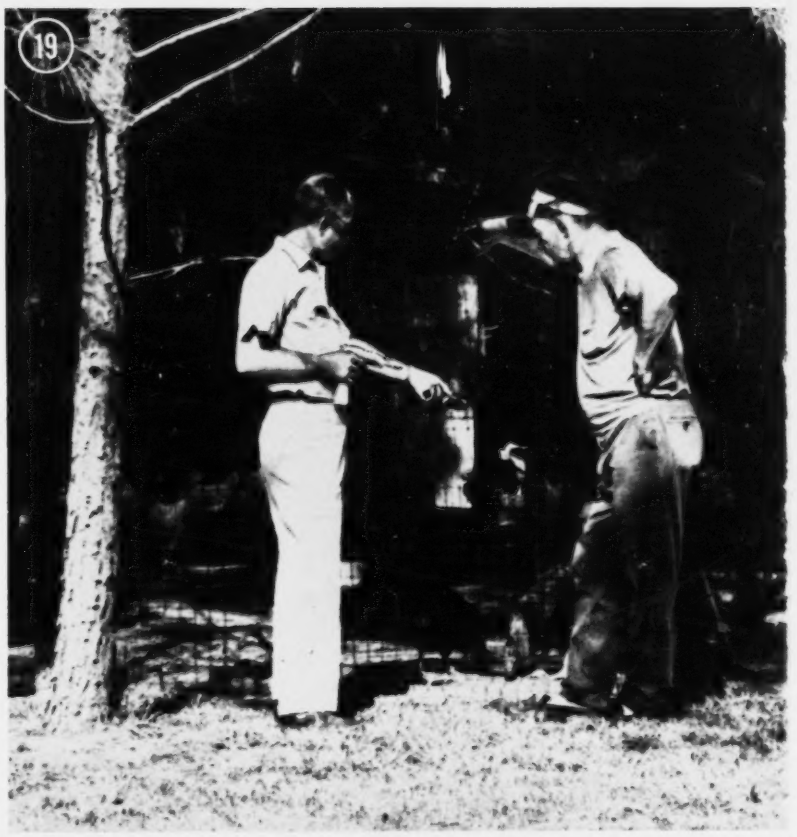
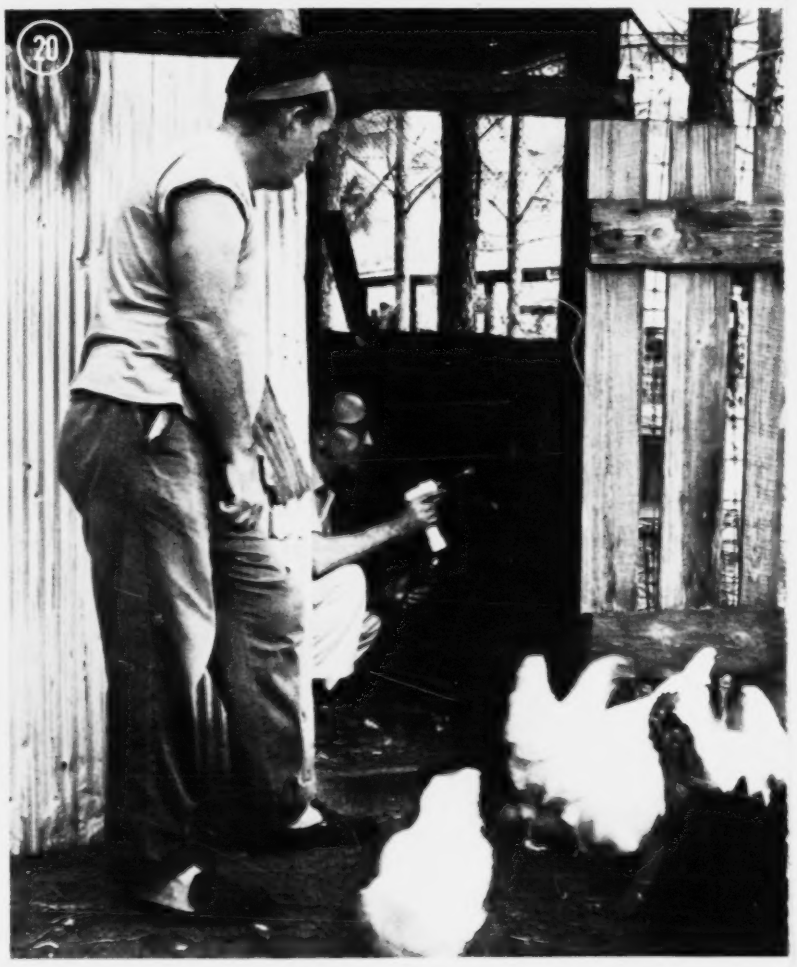
Culverts and bridges (Figure 18) can be located by examining the map for roads over streams or ditches. Chickens can usually be seen or heard while driving slowly through a section. If none are seen, stopping in the middle of a block and looking in back yards or questioning residents may yield locations of chicken houses (Figures 19, 20). Permission must always be obtained from the residents before entering a premise to collect mosquitoes.

During this general search, small numbers of mosquitoes may be caught in many different collecting sites. To test these most economically in the laboratory, all mosquitoes captured in a section may be pooled and labeled with the names of two streets which intersect centrally in the section. Larger collections of mosquitoes may be labeled with a specific address (Figure 21). Well distributed examples of these better sites can serve as routine collecting stations to be revisited periodically to reveal changes in virus activity and fluctuations in the mosquito population. The





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number of such permanent stations and the frequency of visits to them are determined by the over-all plan of operation. Usually it is best to delay collecting around human habitations until 8:00 a.m. to avoid disturbing the occupants. Collecting becomes progressively less profitable beyond 3:00 or 4:00 p.m. since the mosquitoes begin to disperse by that time.

The mosquitoes collected are transferred to small cages made from one-pint cylindrical ice cream cartons by covering one or both ends with fine meshed netting (Figure 22). A cork-stoppered hole in the side permits access. The cages are covered with a dampened towel and transported to a temporary laboratory. There the mosquitoes may be held alive for 24 hours for partial digestion of blood meals to permit inactivation of antibody which might be contained, or they may be killed immediately by freezing on dry ice. After killing, they are sealed in labeled flat-bottomed vials as for light trapped specimens, at approximately −60° C until they are identified and tested.

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Biting Collections

Some day-biting mosquito species are not caught in light traps in sufficient numbers to make up adequate samples for virus tests, nor are they found readily in daytime resting sites. The same is true of some crepuscular and night-active mosquitoes. In these cases human biting collections may be profitable. Most conveniently, two collectors work together. Sleeves and trouser legs may be rolled up, or shirts removed if necessary. The mosquitoes attracted should be captured quickly by aspirator before they have an opportunity to feed (Figure **23**). The individual found to be the more attractive to the mosquitoes serves as the principal bait while the other does the bulk of the collecting. A large animal such as a cow or horse may serve as an effective attractant.

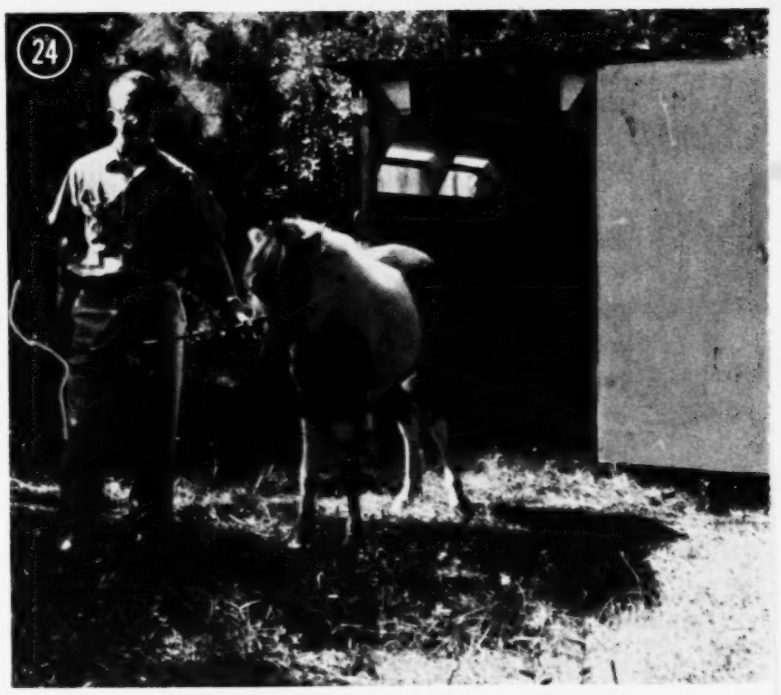
Collecting periods of 15 to 30 minutes per station are generally adequate for routine surveillance during the day or after dark. During the dusk period, however, a single site is usually worked from about a half-hour before sunset until 30 to 45 minutes after sunset since some species bite avidly for only a short time during this period and might be missed if the collectors are traveling between stations.

The mosquitoes caught at each collection site or cluster of associated sites are placed in labeled pint-carton cages until they can be killed by freezing or chloroform, sealed in tubes, and stored on dry ice for transport to the processing laboratory.

Collecting with bait traps

A wide variety of mosquito bait traps has been devised and used in many parts of the world. The basic principle of most of these traps is the same, i.e., the mosquitoes enter an ingress baffle to get to the attractant (usually a live animal) but are unable to find their way back out again. Bait traps are usually more selective than light traps, attracting some species of mosquitoes but not others. This is partly due to the host preference of the mosquitoes and partly to a reluctance of some species to enter the baffles. Bait traps are commonly used in host preference studies but are also valuable for supplementing other methods of collecting or for selectively capturing large numbers of certain species.

Some bait traps, called stable traps, are shed-like and large enough to house an animal the size of a horse or cow. Figure **24** shows a stable trap with a special catching chamber mounted above the entry slot on each side to prevent feeding upon the horse. It



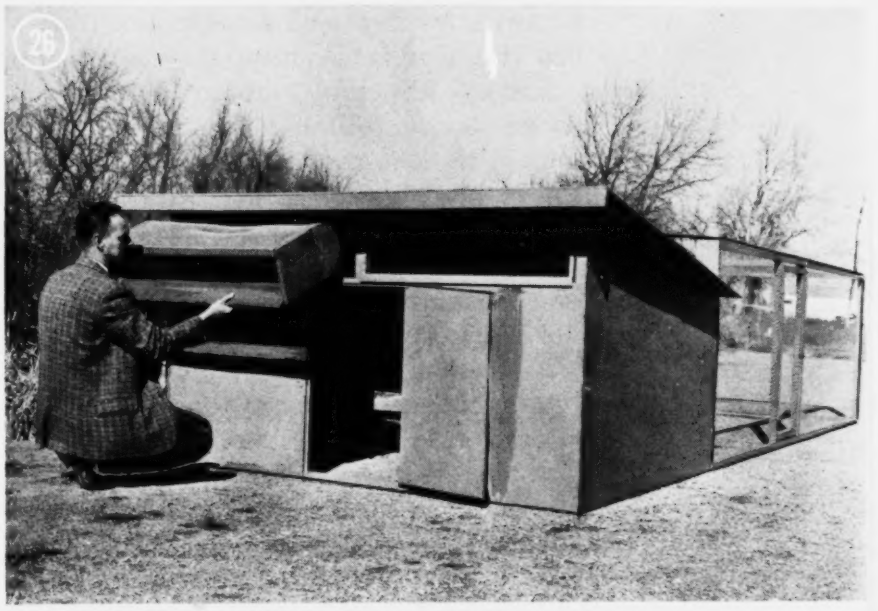
is a more common practice, however, to permit the mosquitoes to enter into the main body of the trap; they are then collected each morning from the walls with an aspirator (Figure **25**).

An effective trap for use in conjunction with a sentinel chicken pen is described by Rainey et al.[4/] A slotted, rectangular capture chamber is affixed to the rear side of the chicken roosting area and captures the mosquitoes upon entry (Figure **26**).

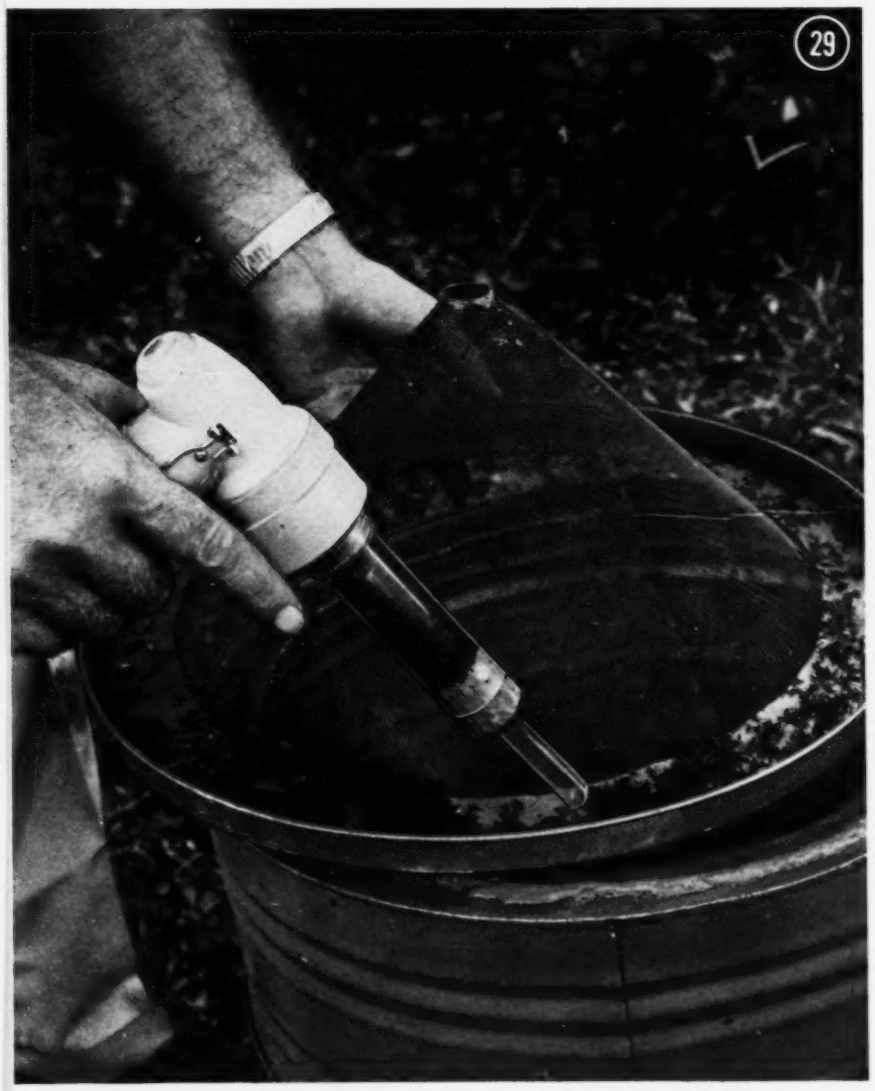
Many small types of traps have been devised which are easily portable and can be baited with various small animals ranging from birds and chicks to reptiles, rodents, and rabbits. Sometimes dry ice as a source of carbon dioxide, serving as an animal simulant, is used. An effective and convenient small type is represented by the lard-can trap of Bellamy and Reeves[5/], which has been used successfully in many field operations. It is a large lard can with a screen cone leading inward from each end. A side door is provided for insertion of a bait animal (Figure **27**). It is hung in a horizontal position from the limb of a tree (Figure **28**). The design may be variously modified to expose the host to mosquito bites or to protect it by screening.

4/ Rainey, M. B., Warren, G. V., Hess, A. D. and Blackmore, J. S., 1962. A sentinel chicken shed and mosquito trap for use in encephalitis field studies. Mosq. News, 22: 337-342.

5/ Bellamy, R. E. and Reeves, W. C., 1952. A portable mosquito bait trap. Mosq. News, 12: 256-258.



Removal of captured mosquitoes from a lard-can trap may be accomplished in several ways, but one especially applicable to field conditions is as follows: First remove the bait animal through the side door; then stand the trap on end, lid uppermost. With a household hand sprayer of the insecticide type, spray chloroform into the top of the can in sufficient quantity to anesthetize the mosquitoes. Next, flip the can over endwise and bump it against the ground, lid end down. This jars the mosquitoes loose from the screen and onto the peripheral area of the lid. The can is then lifted off the lid, and the mosquitoes aspirated (Figure 29) and placed into pint carton cages for recovery and later processing. If a mouth aspirator is used, be certain that the chloroform fumes have dispersed before drawing up the mosquitoes.



PROCESSING MOSQUITOES IN THE LABORATORY

Facilities for processing field-collected materials

One of the problems of greatest concern in the processing of field-collected mosquitoes in the laboratory is to eliminate faulty techniques or conditions which might cause cross-contamination. To solve this problem at CDC a separate laboratory is used exclusively for the sorting, identification, grinding, centrifugation, and storage of the field specimens. No materials known to be virus-infected are permitted in this room. The basic equipment provided, used for no other purpose, includes a refrigerated centrifuge, refrigerator, –60° C mechanical freezer, dissecting microscopes and a chill table for sorting mosquitoes.

All virus work on virus strains isolated, such as passages and virus identification procedures, is done in separate facilities. Even then, field isolates are not handled concurrently with known infected experimental material.

This same caution applies to animal rooms in which the suckling mice are inoculated and kept. Preferably "primary" inoculations (those of field suspensions) are placed in rooms separate from "passage" inoculations, which of course are much more likely to be infected with virus. Mice inoculated experimentally with known viruses are never placed in the same room with field material but are relegated to "hot" areas.

Identification and pooling

Mosquitoes must be accurately identified by species before they are tested. Since it is neither economically feasible nor necessary to test each mosquito separately, they are pooled by species. But first, before identification is commenced, a decision is made as to which collections are closely enough associated in space and time to be grouped together for sorting and pooling purposes. This decision is based mainly upon the distances between collecting sites, the ecological differences or similarities in the various sites, and the degree of precision desired in

determining the time of mosquito infection, should viruses be found present. Generally, collections from closely associated sites over 1- to 2-week collection periods can be grouped without serious loss of specific information. This grouping is done in the interests of economical pooling of the individual species for testing. When several collections are lumped together, more of the mosquito pools, particularly of the minor species, can be made up to maximum size. Since each pool is inoculated into a litter of suckling mice valued at approximately $1.50, conscientious pooling is not only advisable but usually an absolute necessity.

The number of mosquitoes making up individual test pools may range from a single mosquito, if that happens to be the only specimen of a particular species taken, to a maximum of approximately 100. The maximum pool size at the Communicable Disease Center is arbitrarily set at 100 for average sized mosquitoes and fewer for the large mosquitoes such as *Psorophora confinnis* (L.A.) and *P. ciliata* (Fab.). Very small mosquitoes such as the *Uranotaenia* species are processed in pools of up to 200 each. The maximum figure is flexible and can be lowered if a high field infection rate is expected. For example, if as many as 1 of each 5 pools of 100 of a particular species are found infected, the pool size is reduced to 50 in subsequent tests to decrease the possibility of double infections.

Males are generally discarded unless saved for terminalia examination to confirm identification of associated females.

The mosquitoes to be sorted are removed from the freezer one or two tubes at a time and thawed in the hand or by laying on a table top for a few minutes. They are then spilled out onto the inverted lid of a glass 14.5 cm Petri dish lined with a single thickness of slightly moistened filter paper. The desired degree of moistness is obtained by wetting the paper with water, pouring off the excess, then blotting as dry as possible with facial tissue or a soft towel. At CDC the dish of mosquitoes is kept cold during sorting by a specially designed chill table, the surface of which is provided with a refrigeration unit.[6/] In the absence of such refrigerating equipment, however, a suitable cold surface can be prepared by placing a 1-in. thick slab of dry ice in a shallow tray about 16-in. square. The surface of the dry ice should be insulated with a cloth towel and enough thicknesses of paper toweling to prevent refreezing of the specimens; the surface temperature of the mosquito dish should be approximately 3° to 5° C. Flatsided quart-sized frozen refreezant cans or pliofilm bags of cracked water-ice can also be used as sorting surfaces. When using the cracked ice, it is advisable to provide each bag with a vent tube to prevent ballooning as the ice melts.

The mosquitoes are sorted according to species with fine-tipped forceps into smaller Petri dish halves (55 to 60 mm diameter), similarly lined with damp filter paper and conveniently arranged semicircularly on the cold surface around the large sorting dish (Figure 30). To speed up the mosquito handling, the initial sorting is done mostly by naked eye by skilled technicians, trained to distinguish the different species by macroscopic characters. Periodically, whenever

6/ Sudia, W. D., Chamberlain, R. W. and Collier, M., 1965. The CDC entomological chill table, a refrigerated unit for use in processing mosquitoes for virus isolation studies. Mosq. News, 25 (4):385-389.

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ARTHROPOD POOLING RECORD

Date Inoculated 9/1/64

Location Waycross, Georgia

Date Ground 8/26/64

Lab. No.	Field Data	Trap Nites	Species	IBM Code	Red	Black	Gravid	Unfed ♀	Total ♂	Total ♀	Chicks	Baby Mice	3 Wk Mice	TC	Virus Ident.	
GW4-7ag	Harris Farm	9	An. quad.					1		1		–				
7ah	LT 1,2,3		C.(Mel.) sp.				1	2		3		–				
7ai	8/7,8,9/64		A. vexans					100		100		–				
7aj			A. dupreei					25		25		–				
7ak			A. canadensis					5		5		–				
7al			U. sapphirina					200		200		–				
7am			P. confinnis				1	2		3						reinoc
7an			A. mitchellae		1*			6		7		–				
7ao			U. sapphirina					93		93		–				
7ap			A. vexans					47		47		–				
GW4-8a	Harris Farm	9	A. atl-form					100		100		+			Calif.	
8b	LT 4,5,6		C. melanura					50		50		+			EEE	
8c	8/7,8,9/64		An. cruciens				3	97		100		+			Tensaw	
8d			"		1*	3		38		42		–				
8e			C. melanura					50		50		–				
8f.			A. vexans					33		33		–				
8g.			U. sapphirina					134		134		–				
8h			C. nigripalpus			2	3	23		28		–				
8i			A. atl-form				1	88		89		–				
8j			C. melanura			1	1	32		34		–				
	* saved for precipitin test.															

a maximum pool size is reached, mosquitoes are listed on an Arthropod Pooling Record sheet (Figure **31**), transferred to a clean, unlined, 10 cm Petri dish, and the lid given a code number with glass-marking ink or a soft wax pencil (Figure **32**). These dishes are accumulated in a refrigerator at approximately 4° C for up to 4 hours until their contents are checked for accuracy against the pooling record sheet by an entomologist (Figures **33**, **34**). This checking is done with a dissecting microscope, the stage of which is kept cold by resting it upon a chilled surface.

The pooling record sheet provides for recording the unfed (non-engorged), gravid, and blood-engorged (red or black) specimens separately, although they may be ground up in the same pool. However, the abdomens of the more freshly engorged (red) specimens are removed with a clean, sharp scalpel, placed in labeled tubes and saved for later precipitin testing to determine the host fed upon. The remaining portions of these mosquitoes are returned to the pool. If the numbers of freshly engorged mosquitoes are great, or if their abdomens are not removed, they are tested in separate pools which are smaller than those for the other feeding categories. This is done to minimize the possibility of neutralizing virus of an infected mosquito pool by antibody which might be present in the host blood.

The importance of keeping the mosquitoes chilled while sorting cannot be too strongly emphasized. Best knowledge indicates that most arboviruses are highly unstable in dead mosquitoes at room temperature. In view of the considerable effort and expense of obtaining and testing the specimens, one should not take chances of losing everything by careless handling. Not only will virus be lost, but an erroneous picture of the infection rate in the survey area will be obtained which may lead to epidemiologic misinterpretation.

The importance of double-checking the identifications must also be stressed. Regardless of the skill of the initial sorter, mechanical errors invariably occur. Even if a single person is responsible for all mosquito identifications, he should check his own poolings prior to their being ground for testing. The seriousness of misidentification may be extreme, as a virus isolation from a misidentified species may send your own or other research groups after faulty leads concerning vector-virus-host relations.

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Grinding, centrifugation, and storage of suspensions

A supply of sterile diluent is made up each day; however, any left over can be placed in the refrigerator and used again the next day but is not kept beyond that time. It consists of 25 percent normal rabbit serum in 0.05 M phosphate-buffered distilled water, pH 7.6 to 7.8, and contains 1.6 mg of streptomycin sulfate and 1,000 units of sodium penicillin G per ml. The high proportion of serum gives this diluent excellent virus stabilizing properties. The formula for its preparation is as follows:

Penicillin working stock: To 1,000,000 unit bottle, add 10 ml 0.05 M phosphate buffered water (pH 7.8) to produce working stock containing 100,000 units ml.

Streptomycin working stock: To 5.0 gm bottle add 12.5 ml buffered water (pH 7.8) to yield a 400 mg/ml concentrated stock. To prepare working stock containing 40 mg/ml, add 54 ml buffered water to 6.0 ml of the concentrated stock.

For 25 percent normal rabbit serum diluent:

25.0 ml normal rabbit serum
1.0 ml penicillin (100,000 units/ml)
4.0 ml streptomycin (40 mg/ml)
70.0 ml buffered water (pH 7.8)
100.00 ml yield

A double set of labels are prepared for each pool using ½-in. adhesive tape and a ballpoint pen with waterproof ink. Time can be saved by using a rubber stamp for the parts of the labels which are repeated. The labels are placed on sterile, corked Kahn tubes, one pair per tube, and the tubes lined up in order in a rack. The corks are removed and placed temporarily into a clean Petri dish. With an automatic pipette, 2 ml of the 25 percent rabbit serum diluent are dispensed into each of the tubes, and the corks replaced (Figure **35**). The rack of tubes is then placed in a shallow pan of ice.

Five Petri dishes of the identified mosquitoes are removed from the refrigerator at a time. These are lined up in order on a towel-covered work table, about 6 inches apart; in front of each a chilled, sterile 3-in. O. D. mortar is placed. Then, starting from left to right, the mosquitoes are dumped into their respective mortars and the labeled lids again realined with the mortars to prevent confusion as to identity (Figure **36**). Any mosquitoes

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which adhere to the dish are dislodged with a sterile applicator stick, which is then discarded. Two or three drops of diluent are dispensed by dropper bottle into each mortar (Figure **37**), followed by a small amount of powdered Alundum* as abrasive (Figure **38**). In order, each pool is ground well in the small amount of diluent to make a smooth paste. Do not short-cut the grinding. Then the 2 ml of diluent are dumped from the first Kahn tube into the first mortar, the label checked against that on the Petri dish lid to avoid error, and the grinding continued (Figure **39**). When a smooth suspension is obtained, it is poured back into the same Kahn tube (Figure **40**), which is replaced in the iced rack. This procedure is repeated until all of the suspensions are ground. The same amount of diluent (2 ml) is used for each pool, regardless of whether it contains a minimal or maximal number of mosquitoes. The 2 ml volume is convenient to work with, neither restricting reinoculations nor unduly diluting the virus in an infected pool.

*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health, Education, and Welfare.

The above method of predispensing the diluent into the Kahn tubes eliminates the danger of contaminating the entire diluent supply, as can happen when the diluent is pipetted directly to each mortar from a common flask at time of grinding.

The suspensions are spun for 30 minutes at 3000 RPM (1700 X *g*) in a 60-place angle head in a refrigerated centrifuge (Figure **41**). At completion of centrifugation each supernate is poured into a clean corked Kahn tube (Figure **42**) and the two labels transferred. Use caution in handling the stoppers or the fingers may become contaminated; never touch the lip of a tube. The tubes are then placed in pint-sized cylindrical ice cream cartons, the lids of which are labeled as to contents, and stored in a mechanical freezer at −60° C until the suspensions are inoculated intracerebrally into suckling mice (Figure **43**). If preferred, the inoculations can be performed immediately after the centrifugation, before storage. However, there is no evidence that the extra freeze-thaw decreases in appreciable degree the virus titer of an infected suspension, provided the 25 percent serum diluent is used. Also, suspensions have been stored for a year with little or no indication of virus loss.

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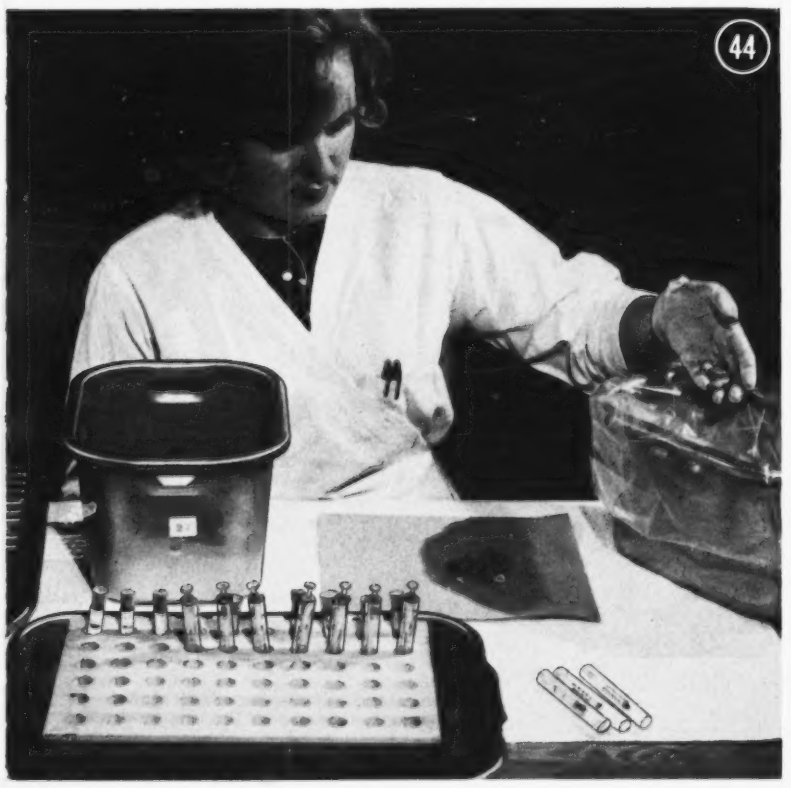
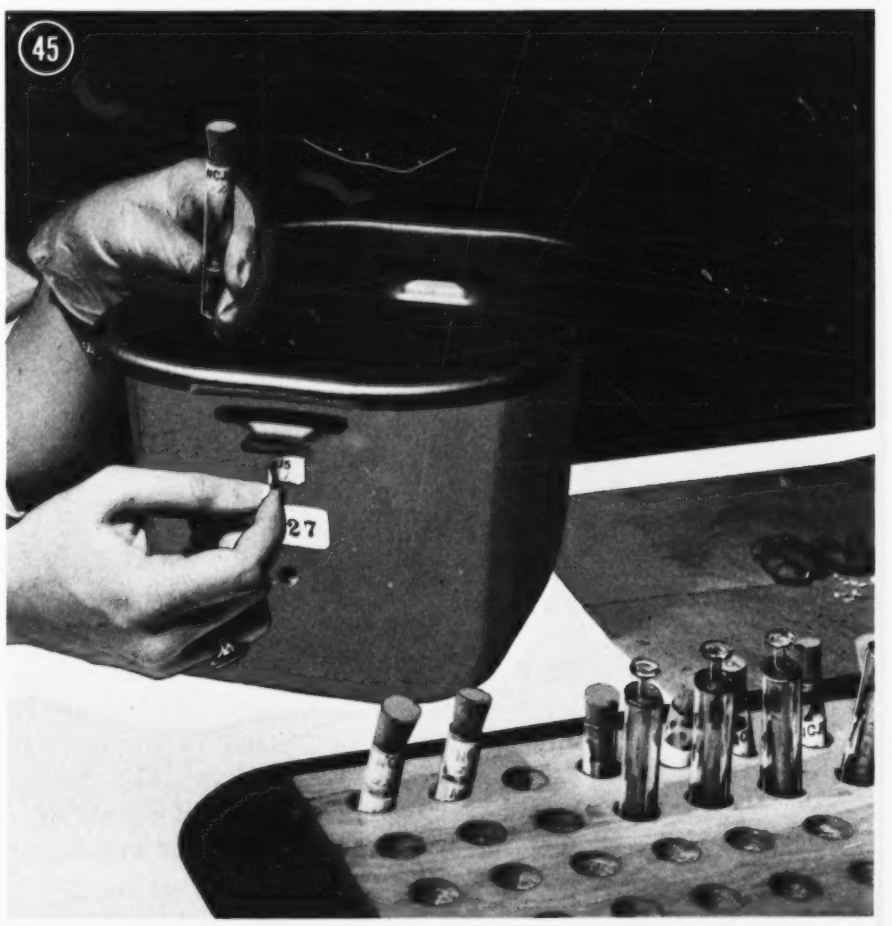
Animal inoculation and observation

Suckling mice are used for the initial isolation of arboviruses from the mosquito pools, in the present absence of a single tissue culture system approaching their broad susceptibility. Weanling mice, although satisfactory for some arboviruses, fail to demonstrate the broad susceptibility required for general arbovirus survey purposes. Pregnant female mice are placed in separate mouse cans when they are nearly ready to litter and are checked each morning. The new litters and their mothers are dated and set aside as "zero days old," to be used on day 1 or day 2. They can be inoculated on the day of birth, but the chance of their being destroyed by their mothers is somewhat increased. They are also sometimes used when three days of age; however, the resistance of mice to some of the arboviruses increases with age, so this practice is ordinarily reserved for Monday inoculations, to use the litters remaining from the previous Friday. Mice older than three days are never used to attempt field isolations where the type of virus to be encountered is unknown.

Each litter is culled to six baby mice (Figure **44**). One of the tube labels is placed on the mouse can (Figure **45**). The mice are each inoculated intracerebrally with 0.02 ml of the mosquito suspension, using a ¼-ml tuberculin syringe and ¼-in. 27-gauge needle. A separate syringe and needle is used for each mosquito pool. Syringe filling (Figure **46**)





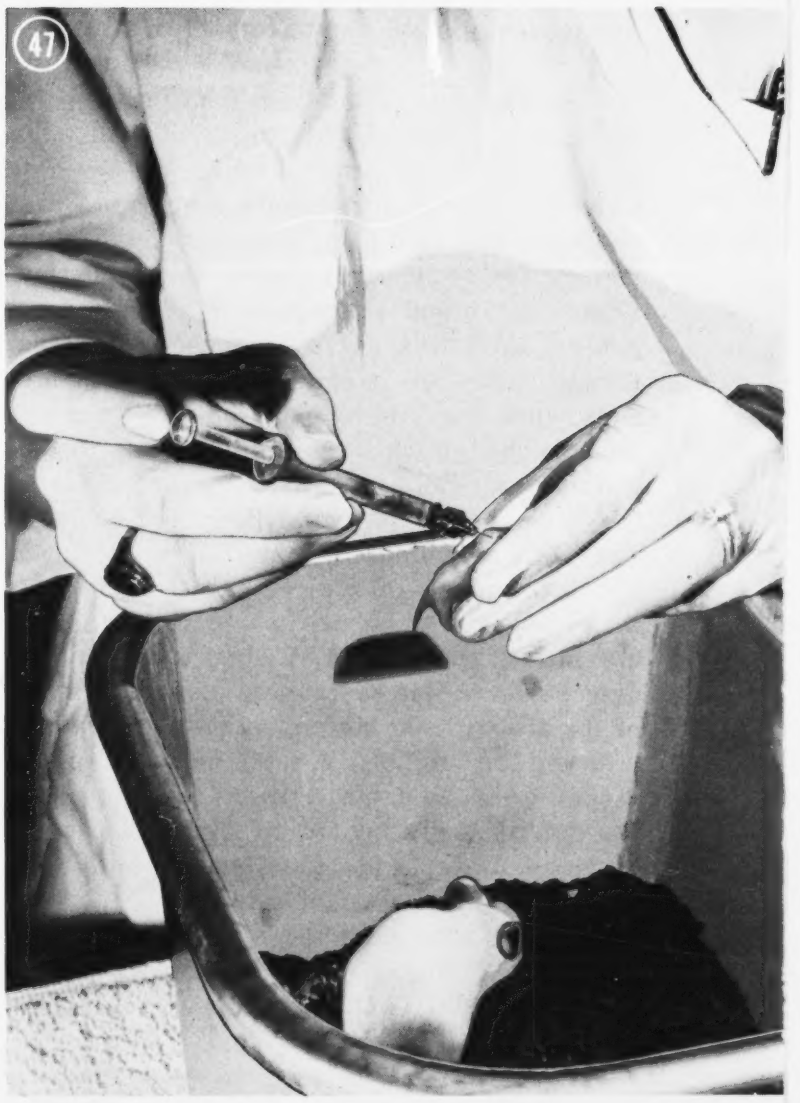
and the mouse inoculations (Figure 47) are performed over the mouse can to minimize contamination of the table area. Special caution is observed to avoid touching the tube lip, plunger shaft, or needle hub since cross-infections from one litter to the next may occur during inoculation if the hands become contaminated. The hands are braced against the edge of the can for steadiness. A disposable pliofilm glove is worn on the hand which holds the mice during inoculation. The tubes of mosquito suspensions are kept in a rack in a shallow pan of ice during the animal room operations.

The inoculated mice are generally placed back into the can with their own mother; however, some technicians pool all the young and place them with mothers at random after inoculation with good success. In fact, the mothers of litters up to a week old may be used by discarding their own young and replacing them with 1- or 2-day-old mice thinned from other litters.

The test mice are checked each morning for 12 to 14 days, using 10-inch forceps (Figure 48). The forceps are alternated frequently with another pair in a jar of disinfectant, especially when an apparently infected mouse is touched. The disinfectant is blotted off with a paper towel before use. The mouse count and symptoms are recorded on an Animal Inoculation Record sheet, using standardized abbreviations: M, missing; E, eaten by mother; D, dead; Pa, paralyzed; Pr, prostrate; and Co, convulsing. Additional designations may also be helpful, such as Tw (twitchy), Wo (wobbly), and Pa? (questionably paralyzed or with indefinite symptoms, to be observed closely on future checkings). When symptoms begin to occur, it is wise to check the mice again in the late afternoon. A dash (–) in the checking column indicates normality.

A sample Animal Inoculation Record sheet is shown in Figure 49. These are kept in loose leaf binders in page order. For convenience, inoculation sheets of field suspensions are kept in a "Primary" book; those of passages are kept in a separate "Passage" book.

The dead mice and those showing definite signs of infection are saved for passage or later reference. Usually the mice with severe symptoms, such as general paralysis



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ANIMAL INOCULATION RECORD

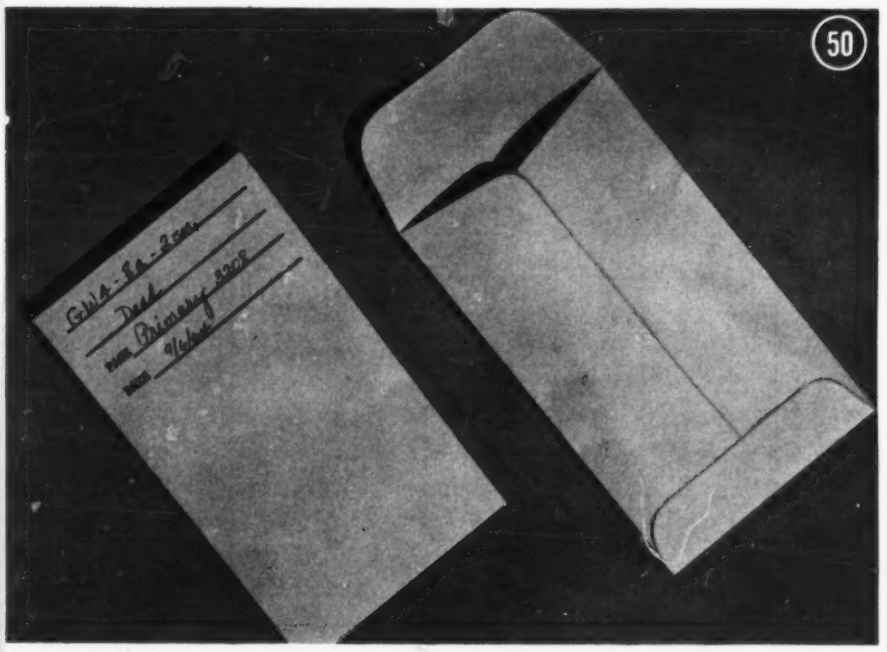
Date: 9/1/64 Page: 3208

Animal, route, am't inoc.: SM. ie 0.02 cc

Diluent: 25% NRS + A Exper.: Field Studies Category: Primary

MATERIAL INOC.	DATE OF CHECKING 2	3	4	5	6	7	8	9	10	11	12	13	14	
GW4 - Tal	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	
	-	-	-	-	-	-	-	-	-	-	-	-	-	
From Bk:___ page:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cultures	-	-	-	-	-	-	-	-	-	-	-	-	-	
Br:___ Susp:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
GW4 - Tam	-	-	-	-	-	-	M							
	-	-	-	-	-	-	-	-	M					
	-	-	-	-	-	-	-	-	-	-	-	-	-	
From Bk:___ page:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cultures	-	-	-	-	-	-	-	-	-	-	-	-	-	p.3217
Br:___ Susp:___	-	-	-	-	-	-	-	-	-	-	-	-	-	lst. reinoc
GW4 - Tam	M													
	E													
	-	-	-	-	-	-	-	-	-	-	-	-	-	
From Bk:___ page:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cultures	-	-	-	-	-	-	-	-	-	-	-	-	-	
Br:___ Susp:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
GW4 - 8a	-	-	-	-	M									
	-	-	-	-	D	saved 9/6/64 5657 Virus + Calif								
	-	-	-	-	wo	M								
From Bk:___ page:___	-	-	-	-	wo	D saved 9/7/64								
Cultures	-	-	-	-	-	Pa/K } saved 9/7/64								
Br:___ Susp:___	-	-	-	-	-	Pa/K }								
GW4 - 8b	-	D }	5658 Virus + EEE											
	-	D }	saved 9/3/64											
	-	D												
From Bk:___ page:___	-	D												
Cultures	-	M												
Br:___ Susp:___	-	M												
GW4 - 8c	-	-	-	-	-	-	-	-	D	saved 9/10/64 5659 Virus + Tensaw				
	-	-	-	-	-	-	-	-	Pa/K	saved 9/10/64				
	-	-	-	-	-	-	-	-	-	-	-	-	-	
From Bk:___ page:___	-	-	-	-	-	-	-	-	-	-	-	-	-	
Cultures	-	-	-	-	-	-	-	-	-	-	-	-	-	
Br:___ Susp:___	-	-	-	-	-	-	-	-	-	-	-	-	-	

or prostration, and those very recently dead, contain more virus in their brains than mice with lesser symptoms. The sick mice are killed by brief exposure to chloroform fumes in a killing jar, then are placed in suitably labeled end-flap 3- x 5-in. manila envelopes. The label indicates the mouse involved, symptom, date, and page in the record book (Figure **50**). The mice saved are stored in date order in a mechanical freezer at –60° C until processed further.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Strict maintenance and sanitation practices are observed in the animal rooms at all times. Ample food and water is constantly available to discourage the mothers from eating their own young. At the close of the observation period, all surviving mice are killed by chloroform. Then they, their bedding material, and any left-over mouse food are incinerated, and the mouse cans and water bottles are sterilized by autoclaving.

Harvesting mouse brains for passage

Periodically, usually at weekly intervals, the Animal Inoculation Record sheets are inspected, and mice are selected from the freezer for brain passage into other suckling mice. A serial code number (passage number) is given each mouse selected, and is recorded both on the Animal Inoculation Record sheet and the mouse envelope. At the same time a passage listing is made out on a Passage List sheet (Figure **51**), associating the passage number with mosquito suspension, mouse source, and record book pages. Columns are also provided for recording results of sterility tests on brain suspensions. This list is valuable in tracking down the laboratory history of material tested since it shows in one place the source, disposition, dates of handling and association with other materials.

Generally all single, scattered "deads" are passed unless the mice are obviously decomposed or have died of some cause easily recognized as foreign to arboviruses. If more than one mouse in a litter dies, in a pattern consistent with virus infection, usually only a single mouse of the group is passed and the remainder held until the outcome is known. If the first mouse passed is negative, a second mouse is passed, provided the symptom or day of death was different from that of the first mouse.

The goal is a clear-cut positive or negative result from each mosquito pool inoculated, but frequently a fully qualified negative test is difficult to obtain. Ideally, a pool is considered negative if all mice inoculated survive for the full checking period, or when dead mice are involved, they are passed with negative results. In many instances, however, non-specific deaths may have occurred so early in the majority of mice in an inoculated litter as to cause the test to be considered unsatisfactory, in which case the original suspension must be reinoculated.

Also, the mother mice may eat some of their young. This is usually not critical if only one or two are eaten on the first day of inoculation; four survivors past the first day are generally considered a legitimate test since few arboviruses kill suckling mice within one day. When missing mice occur at later incubation periods, however, or when an arbovirus is suspected which kills within one day, a strong possibility exists that they were eaten because they were sick. These suspensions must be reinoculated.

Some suspensions appear to be toxic, killing the mice on the first day. If this repeats on second inoculation, and passage fails to indicate presence of a virus, the material should either be diluted 1:10 for

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PASSAGE LIST

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Date listed: 9/12/64 Date ground: 9/13/64 Date inoc: 9/15/64

Date dissected: 9/13/64 Host: SM Route: i.c. Vol: .02cc

Date of Death	Laboratory Designation	Cultures			Record Books From		Record Books To		
		Code	Br.	Susp.	No.	Page	No.	Page	
8/20	F1-282 w-3 SM$_1$-1 SM$_2$-4 SM$_3$	5649			PASS.	631	PASS.	647	
8/22	F1-284 p-4 SM$_1$	5650			PRIM.	3121	"		
8/24	FE 3-45 ai-3 SM$_1$	5651				3128	"		
9/2	FE 3-61 hi-1 SM$_1$	5652				3146	"		
9/1	FE 3-61 lo (1st reinoc) 2 SM$_1$	5653				3167	"		
9/8	GW4-6b-2 SM$_1$-4 SM$_2$	5654			PASS.	634	"		
9/3	GW4-6 cd-1 SM$_1$	5655			PRIM.	3183	648		
9/5	GW4-6 ek-1 SM$_1$	5656				3196	"		
9/6	GW4-8a-2 SM$_1$	5657				3208	"		
9/3	GW4-8b-1 SM$_1$	5658				3208	"		
9/10	GW4-8c-1 SM$_1$	5659				3208	"		

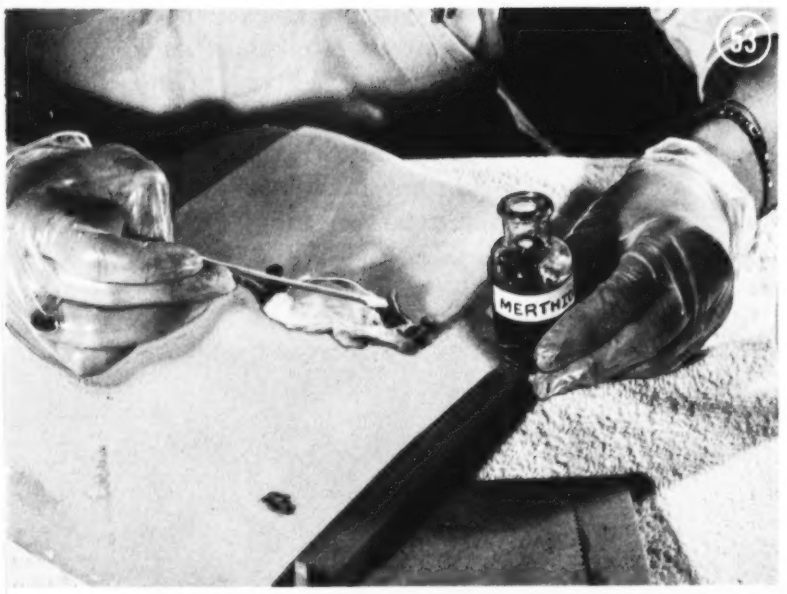
reinoculation or placed in hamster kidney or duck embryo tissue cultures. It may also be inoculated into suckling mice by the subcutaneous or intraperitoneal routes, since most arboviruses which kill by the intracerebral route are also infectious when inoculated peripherally. Mice are less subject to toxicity of materials inoculated peripherally.

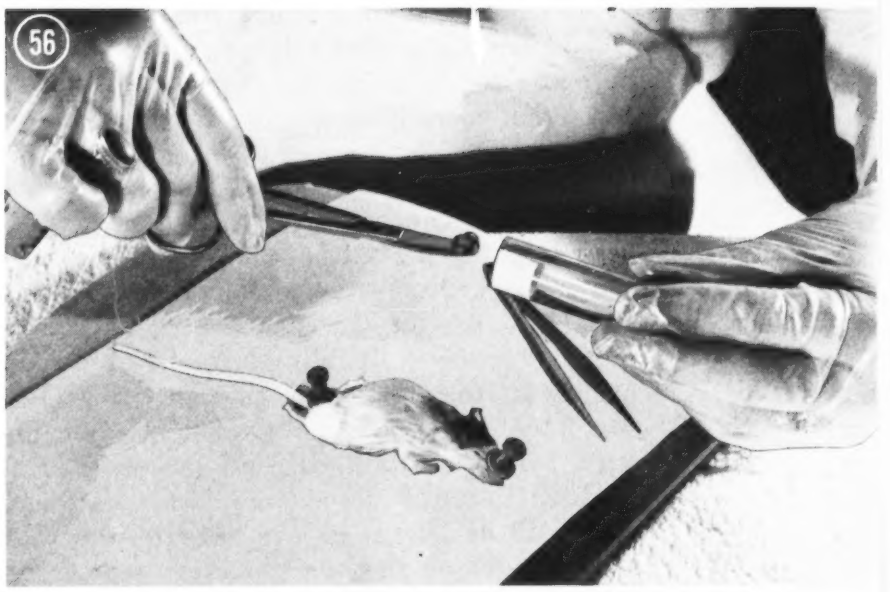
The positive pools are more easily recognized, since one or two passages generally result in all mice dying within two or three days of each other. The brains of some of these mice are used for identification by complement fixation, hemagglutination, and neutralization tests. Some are also saved as stock for future reference. When a mosquito pool has been found to contain virus, the original suspension is generally reinoculated to confirm the validity of the isolation.

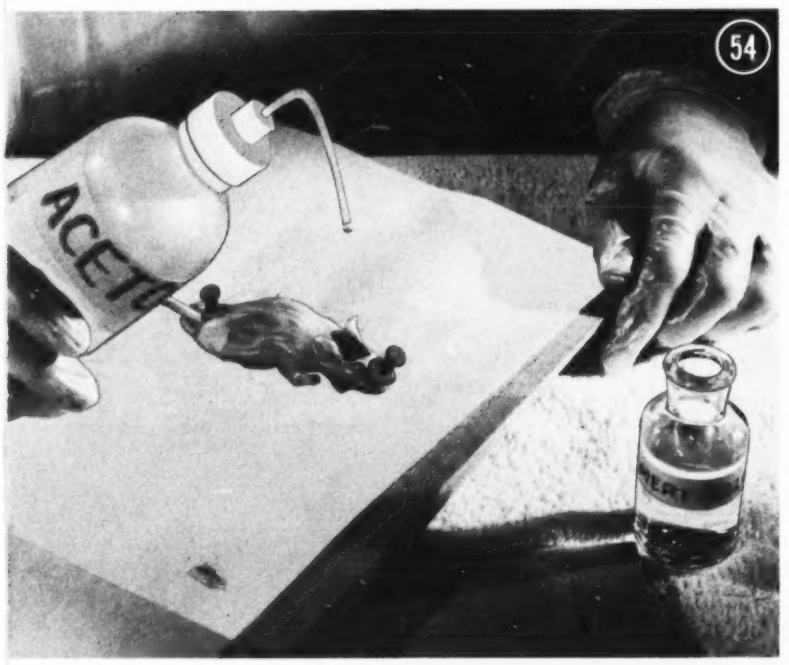
It is during the passaging procedure that laboratory contamination and serious error are most likely to occur. Infected suckling mouse brain tissue often contains very high concentrations of virus; titers of $10^{9.0}$ with some viruses are not unusual. This means that the material could be diluted a billion fold and still contain enough virus to kill suckling mice. In handling viruses in such concentration, considerable caution must be taken to prevent risk to the technician and cross-contaminations between different brain specimens. Carelessness during the preparations can result in apparent "isolations" from associated non-infected suspensions, leading to considerable confusion and serious misinterpretations.

Manipulations should be carried out in a properly exhausted hood. Dissecting instruments (curved iris scissors and 5-in. thumb forceps) are sterilized by boiling for 20 minutes in an instrument sterilizer. Two sets are used for each mouse, one to remove the skin from the cranium and the other to take out the brain. The method described below can be used for either suckling or weanling mice.

The frozen mice are partially thawed, dipped in 2 percent Lysol solution, blotted briefly on paper toweling, and pinned to a softwood dissecting board covered with a paper towel and a piece of butcher parchment. Map pins are used to fasten the mouse down, one placed through the bridge of the nose and another through the base of the tail. The scalp is removed with the first set of instruments (Figure **52**). These instruments are then wiped on a gauze pad and stacked in a jar of 2 percent Lysol disinfectant, to await later sterilization by boiling. The skull cap and adjacent skin are swabbed with 1:1000 Merthiolate and the swab-stick discarded (Figure **53**). A drop or two of acetone are then delivered from a height of about 2 inches by dropper to wash away the excess Merthiolate and to cause rapid drying of the skull surface (Figure **54**). It is dropped from this height to minimize chance contamination of the dropper tip, since the same one is used for each mouse. A second set of instruments is then used to cut off the skull cap and take out the brain (Figure **55**). It is advisable to have the brain only partially thawed or it will be too viscous to handle. Freshly killed mice of course pose no problem in this respect. The brain is transferred to a labeled, sterile, corked, flat-bottomed shell vial (17 X 55 mm) (Figure **56**); then a culture is made by immersing the tip of the scissors into a tube of thioglycollate broth and wiping against the side of the tube. These scissors and forceps are then also set aside to be sterilized. The label on the tube is checked against that on the envelope from which the mouse was taken, since confusion of labels is a serious source of error.



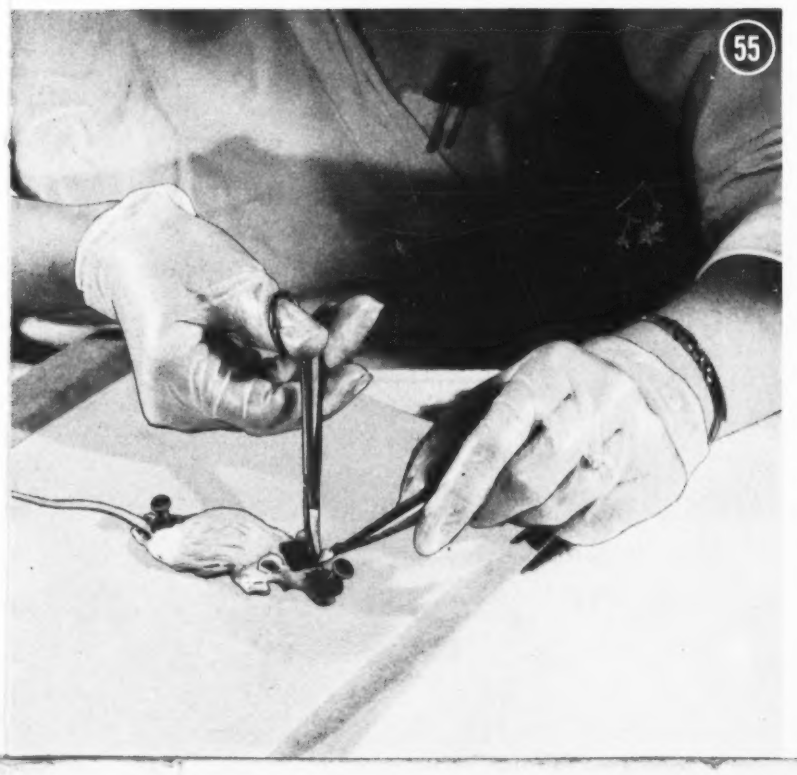




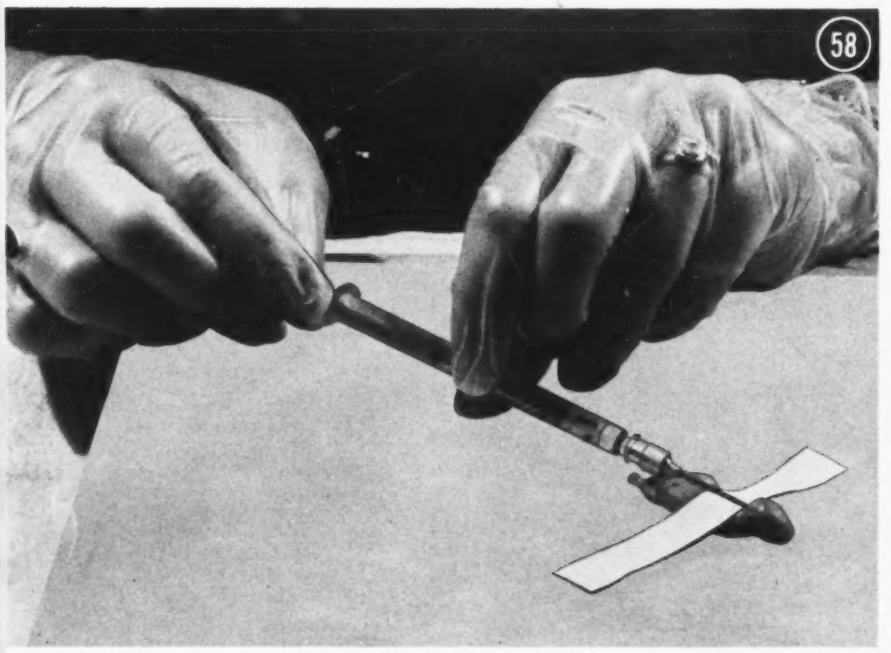
The pins are removed and placed in a container of Lysol solution. The mouse carcass is wrapped in the butcher parchment and paper towel and placed in a plastic bag for disposal. The mouse board receives a new set of covering papers, and sterile instruments are used for the next mouse.

The vials containing the mouse brain tissue are accumulated in a refrigerator during the dissection period. These tissues are then either ground immediately into suspensions or are frozen at –60° C to be ground at a later date.

Another method can be used for suckling mouse brain harvesting which is considerably speedier but which requires special caution to preclude dangerous aerosols. Antibiotic-treated 25 percent rabbit serum diluent is distributed in 2ml amounts into sterile corked Kahn tubes. These tubes are appropriately labeled, placed in a rack, and the rack is set in an ice bath in a safety hood (Figure **57**).



Frozen suckling mice are allowed to thaw completely. With forceps, each mouse to be harvested is placed belly down, head away from the operator, upon a dissecting board covered with a sheet of butcher parchment. A 3-in. length of ½- or 1-in. adhesive tape is placed transversely over the mouse to hold it firmly down onto the paper; the leading margin of the tape should be across the shoulders, leaving the head free. The mouse's record number is double checked against the number of the previously labeled tube. Then the head and neck are swabbed thoroughly with 1:1000 Merthiolate and the swab discarded. Using a disposable 1.0 ml syringe and a 19 or 20 gauge 1-in. needle, the cranium is pierced from its posterior aspect and 0.1 to 0.2 ml of the viscous brain material drawn up (Figure 58). Disposable syringes are used for greater vacuum. The syringe is inserted into the labeled tube but the contents are not yet discharged. Instead about 0.5 ml of the antibiotic-treated diluent is slowly drawn into the syringe along with the brain material (Figure 59). Then, holding the needle below the level of the remaining diluent, all the material in the syringe is slowly ejected *a single time*. No additional drawing-up and discharging should be done since dangerous aerosols will be produced. The syringe and needle are discarded. *Always use a well ventilated hood.* A gentle shaking after replacement of the cork will assure adequate mixing (Figure 60); if virus caused the mouse's death, it will be present in such high concentration that better mixing is not required. The suspension is now ready for centrifugation, described below. Where cultures are desired, these are made by inserting the needle tip into a tube of thioglycollate medium immediately after the brain harvesting, before dilution.


58

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Grinding, centrifuging and passing mouse brain material

Each dissected mouse brain is transferred to a cold, sterile, 3-in. O. D. mortar with a sterile applicator stick (Figure 61) and ground in 2 ml (for suckling mouse brain) or 3 ml (for weanling mouse brain) of the 25 percent rabbit serum diluent to make an approximate 10 percent brain suspension. (Grinding is not required for suckling mouse brains harvested by the syringe technique). Each suspension is poured into a sterile, corked, double-labeled Kahn tube and centrifuged in the cold for 20 minutes at 2500 RPM (1100 X *g*) in an angle-head centrifuge. A separate centrifuge from the one used to spin down the mosquito suspensions is employed to minimize the chance of accidental contamination of primary material. The supernates may be poured off into clean corked Kahn tubes and the labels transferred; however, for routine passages, this step may be skipped. The brain tissue forms such a tight pellet at the bottom of the tube that the clear fluid above may be drawn up into a syringe for inoculation without unduly disturbing it. The suspensions are either inoculated immediately by the intracerebral route into litters of suckling mice or are frozen at –60° C to be inoculated later. If a high proportion of the passages are expected to be virus positive, it is a good practice to intersperse several normal brain suspensions in each inoculation series as a control against careless handling and cross-contamination.

Daily mouse checks are made as before. In the event of apparent arbovirus isolations, freshly dead, prostrate, or paralyzed mice are saved and brains harvested for virus identification procedures.

In a busy laboratory a considerable number of arbovirus isolations may be made from several different field areas concurrently, and following the results of the various inoculations may become cumbersome. The virus isolation progress form shown in Figure 62 provides for convenient recording of data as it develops.

ARTHROPODS OTHER THAN MOSQUITOES

The methods of collection of various other medically important arthropods are many and varied and will not be discussed here. However, the same principles expounded for handling the mosquitoes apply: keep the specimens alive or keep them frozen at dry ice temperature.

Some arthropods, particularly ticks, will often be engorged with vertebrate blood when collected. If the blood is partially digested, there is no laboratory problem. If, however, the blood is fresh, it may still contain some active arbovirus antibody of the host and could conceivably inactivate virus in the pool during the grinding process. It is therefore recommended that freshly engorged arthropods be held alive until partial digestion occurs, to denature any arbovirus antibody present. If this is not possible, they should be tested in smaller pools than ordinarily would be the case, separate from those not containing red blood.

With some arthropods, particularly mites, bird lice and immature ticks, accurate identification is impossible without specially prepared mounts. In these instances, separation as to different kinds is made to the best of one's ability under a dissecting microscope, and a 10 percent sample of each of these kinds is preserved for identification purposes.

The pool size may vary according to the expected infection rate and the size of the arthropod. If the infection rate is high, the pool size must be reduced to prevent double infections in single pools. If the arthropod is

VIRUS ISOLATIONS FROM ARTHROPODS

PAGE _____

TYPE OF ARTHROPOD Mosquitoes COLLECTORS Sudia, Harmston, Newhouse, Johnston INCLUSIVE DATES 9/10/64-10/9/64 SERIES KD-4 AREA Danville, Kentucky

ISOLATION NO.	ARTHROPOD SPECIES	NO. IN POOL (FEED STATUS)	DATE COLLECTED	HOW COLLECTED	COLLECTION SITE	PRIMARY ISOLATION HOST	PRIMARY ISOLATION D/INOC *	PRIMARY ISOLATION ST **	PRIMARY ISOLATION PAGE	REISOLATION HOST	REISOLATION D/INOC	REISOLATION ST	REISOLATION PAGE	PASSAGE HOST	PASSAGE D/INOC	PASSAGE ST	PASSAGE PASS. NO.	PASSAGE PAGE	IDENT.	MISC.
KD4-13a	Culex pipiens	47 (26 black, 21 red)	9/12/64	chicken house	615 Oak St.	SM ***	6/6	7	3901	SM	6/6	7	4140	SM	6/6	5	6645	762	SLE	
KD4-14a	"	39 (21 unfed, 16 gravid, 2 black)	9/13/64	culverts	Earl & High St. sites # 20, 21	SM	3/4	6,7	3943	SM	3/6	7,8	4140	SM	6/6	4,5	6695	772	Flanders	
KD4-47a	"	24 (10 unfed, 10 gravid, 4 black)	9/21/64	store windows	downtown Danville, Ky.	SM	6/6	7	3991	SM	6/6	6,7	4140	SM	5/5	4	6742	781	SLE	
KD4-54b	"	46 (41 black, 3 gravid, 2 unfed)	9/23/64	chicken houses	sites # 1-7	SM	3/6	6	3994	SM	1/5	10	4141	SM	6/6	4	6739	780	Flanders	
KD4-70a	"	50 (42 black, 5 red, 3 gravid)	9/27/64	chicken houses	sites # 1-7	SM	1/6	8	4000	SM	2/6	7,8	4141	SM	6/6	4	6756	785	SLE	

* D/inoc = No. of mice dying over the number inoculated
** ST = survival time in days
*** SM = suckling mice 1-3 days of age

large, only a small number may be ground in 2.0 ml of diluent. It may be advisable in some instances when the arthropod is large to increase the amount of diluent per pool to 5.0 ml. Although an occasional weak virus infection may go undetected because of the additional dilution, the number lost would not ordinarily be great enough to seriously affect the significance of survey data.

The methods already described for laboratory processing of mosquitoes generally apply, except that a greater amount of abrasive powder (Alundum) may be required for the grinding of ticks since they are leathery and tough. Sometimes they are ground while still frozen.

VERTEBRATE BLOOD AND TISSUE

The methods of trapping and bleeding various vertebrates in the field are beyond the scope of this manual and will not be discussed here. However, in view of the fact that the samples obtained are generally processed in association with the arthropod material, the handling of vertebrate samples will be considered briefly.

Whole blood specimens are allowed to clot and the serum is removed. Serum removal is facilitated by use of a small, portable angle-head centrifuge carried with the field equipment. Both the clot and the serum are then sealed and frozen on dry ice for transport to the laboratory, or they may be held unfrozen on wet ice if rapid transport to the laboratory is possible. The clot can be ground later in two to four times its volume of diluent, centrifuged, and used for virus isolation tests, thus conserving the serum for serologic tests if it is in short supply.

Blood specimens diluted in part with antibiotic-fortified 25 percent normal rabbit serum diluent may be kept on wet ice for several days. If tests are for virus only, or if the only serological tests to be run are neutralization tests, the whole mixture may be frozen. If, however, other serologic tests are contemplated, the specimens should be centrifuged and the diluted serum removed before freezing. Generally the small clot is discarded.

The carcasses of small rodents are placed in plastic bags, labeled, and frozen to return to the laboratory. There they are stored at −60° C in a mechanical freezer until ready to dissect. A small piece of brain tissue weighing approximately 0.2 gm is removed from the thawed animal with sterile precautions, ground in 2.0 ml of the 25 percent rabbit serum diluent, centrifuged, and inoculated into suckling mice for testing. A similar pool is made of combined small pieces of heart, liver, and spleen.

The other laboratory isolation procedures are similar to those already described in detail in the mosquito section.